A NEW ESSENTIAL DOWNSTREAM COMPONENT OF THE WINGLESS SIGNALING PATHWAY AND THERAPEUTIC AND DIAGNOSTIC APPLICATIONS BASED THEREON

The present invention relates to a new essential downstream component of the Wnt/Wingless (Wnt/Wg) signaling pathway and therapeutic and diagnostic applications based thereon. The invention relates to nucleotide sequences of the *Drosophila melanogaster legless* (*lgs*) gene, of its encoded proteins, as well as derivatives (e.g., fragments) and analogues thereof. The invention further includes vertebrate and invertebrate homologues of the Lgs protein, comprising proteins that contain a contiguous stretch of amino acids with similarity to the Drosophila *lgs* gene. The invention further relates to the function of the Drosophila and the human Lgs proteins. Methods for producing the Lgs proteins, derivatives and analogs, e.g. by recombinant means and antibodies to Lgs are provided by the present invention. In addition, the invention also relates to therapeutic and diagnostic methods and compositions based on Lgs proteins and nucleic acids or fragments thereof.

Background of the invention

Wnt genes encode a large family of secreted, cystein rich proteins that play key roles as intercellular signaling molecules in a wide variety of biological processes (for an extensive review see (Wodarz and Nusse 1998)). The first Wnt gene, mouse *wnt-1*, was discovered as a proto-oncogene activated by integration of mouse mammary tumor virus in mammary tumors (Nusse and Varmus 1982). Consequently, the involvement of the Wnt pathway in

cancer has been largely studied. With the identification of the *Drosophila* polarity gene *wingless* (*wg*) as a *wnt-1* homologue (Cabrera, Alonso et al. 1987; Perrimon and Mahowald 1987; Rijsewijk, Schuermann et al. 1987), it became clear that *wnt* genes are important developmental regulators. Thus, although at first glance dissimilar, biological processes like embryogenesis and carcinogenesis both rely on cell communication via identical signaling pathways. In a current model of the pathway, the secreted Wnt protein binds to Frizzle cell surface receptors and activates the cytoplasmic protein Dishevelled (Dsh). Dsh then transmits the signal to a complex of several proteins, including the protein kinase Shaggy/GSK3 (Sgg), the APC tumor supressor, the scaffold protein Axin and β-Catenin (β-Cat), the vertebrate homologue of Drosophila Armadillo. In this complex β-Cat is targeted for degradation after being phosphorylated by Sgg. After Wnt signaling and the resulting down-regulation of Sgg activity, β-Cat (or its Drosophila homologue Armadillo) escape from degradation and accumulate into the cytoplasm. Free cytoplasmic β-Cat translocates to the nucleus by a still obscure mechanism, and modulates gene transcription through binding the Tcf/Lef family of transcription factors (Grosschedl R 1999). Mutations of β-Cat itself or of negative regulatory elements, like APC and Axin, that lead to nuclear accumulation of β-Cat and consequently to constitutive activation of the Wnt pathway have been observed in many types of cancers, including colon, skin and breast cancer (Barker N 1999; Morin 1999; Potter 1999; Roose and Clevers 1999; Waltzer and Bienz 1999). Currently, there are no known therapeutic agents effectively inhibiting β-Cat transcriptional activation. This is partly due to the fact that many of the essential components required for its full activation and nuclear translocation are still unknown. Consequently, there is

an urge to understand more about this pathway to develop effective drugs against these highly malignant diseases.
In order to identify new components required for Wingless (Wg) activation we used a *Drosophila* genetic approach. Specifically, we screened for dominant suppressors of the rough eye phenotype caused by a transgene that drives ectopic expression of Wg, the *Drosophila* homologue of Wnt, during eye development. Three genes were identified: the *ß-cat* homologue *armadillo* (*arm*), the *tcf/lef-1* homologue *pangolin* (*pan*) and *legless (lgs)*, a completely new gene. We subsequently cloned *lgs* and confirmed its *in vivo* requirement for Wg signal transduction in embryo and in developing tissues. Epistasis experiments revealed that Lgs is at the same level or downstream of Arm. In addition, we found that the Lgs protein binds to and translocates to the nucleus with Arm in mammalian cells. Biochemical experiments confirmed the binding of Lgs to Arm. Lgs forms a tri-molecular complex with Pan and Arm and enhances the transcriptional activity of the complex.
Sequence homology search using the Blast search tool at NCBI revealed at least two human proteins sharing short amino acids domains with up to 66% sequence identity with *Drosophila* Lgs (dLgs). One of them, hLgs/Bcl9, has been previously implicated in B cell malignancies (Willis, Zalcberg et al. 1998). The other, hLgs-1, is a completely new gene. Several Expressed Sequence Tags (EST) could be found for both human homologues in the public human genome database, demonstrating the presence of their gene products in human normal and tumor tissues. Subsequent genetic and biochemical experiments confirmed the functional homology of hLgs to dLgs. Particularly, hLgs/Bcl9 not only binds to β-Cat and its Drosophila homologue Armadillo (Arm), but is also able to substitute for lack of dLgs during fly development. Furthermore, point mutations or deletions in the homology domains between dLgs and hLgs disrupt Lgs function, highlighting the essential role of these evolutionary conserved domains.

Lgs thus represents an exquisite target for all the diseases caused by the over-activation of the ß-Cat/Tcf complex.

Summary of the invention

The present invention relates to the discovery of a novel family of proteins present in insects and vertebrate organisms, referred to hereinafter as "Legless (Lgs)" proteins. These proteins play an essential role in the Wnt/Wg signaling pathway, and thus in the formation and maintenance of spatial arrangements and proliferation of tissues during development, and in the formation and growth of many human tumors.

In general, the invention relates to nucleotide sequences of the *Drosophila melanogaster lgs* gene, of its encoded protein, as well as derivatives (e.g., fragments) and structural and functional analogs thereof. The invention further includes the predicted nucleotide and protein sequences of a human *lgs* homologue, *hlgs-1*, and the use of another human Lgs homologue hLgs/Bcl9 (Willis, Zalcberg et al. 1998), to modulate or diagnose diseases related to the Wnt signaling pathway.

In one embodiment, the isolated nucleic acid comprises a sequence having at least 50% sequence identity, preferably at least 70% sequence identity, more preferably at least 80% sequence identity, even more preferably at least 90% sequence identity, yet even more preferably at least 98% sequence identity, and most preferably 100% identity to (a) a nucleic acid molecule encoding a Lgs polypeptide having the sequence of amino acid residues from 1 to 1484 of Figure 2, or (b) the complement of the nucleic acid molecule of (a).

In another embodiment, the isolated nucleic acid containing a sequence having at least 30% sequence identity, preferably 50% sequence identity, more preferably at least 70% sequence identity, even more preferably 90% sequence identity, yet even more preferably 95% sequence identity to (a) a nucleic acid molecule encoding a human Lgs polypeptide of figure 10 or (b) the complement of the nucleic acid molecule of (a).

In a further embodiment, the isolated nucleic acid comprises a sequence with a low overall sequence identity but shows a sequence identity of at least 30%, preferably at least 50%, more preferably at least 70%, even more preferably at least 90% and most preferably 100% in the evolutionary conserved domains described in Figure 7.

In yet another embodiment of the present invention isolated nucleic acids encode polypeptides having a function resembling that of the *lgs* gene products.

In another embodiment, the invention relates to a fragment of the *Drosophila* or human *lgs* nucleic acid sequences that can find use as hybridization probe. Such nucleic acid fragments are about 18 to about 100 nucleotides in length, preferably from about 20 to about 60 nucleotides in length, most preferably from 20 to 50 nucleotides in length and can be derived from the nucleotides sequences shown in Figure 2 and Figure 10.

In another aspect, the invention provides a vector comprising a nucleic acid molecule encoding vertebrate or invertebrate Lgs proteins or a fragment thereof. The vector can comprise any of the molecules or fragments thereof described above.

The invention also includes host cells comprising such a vector. By the way of example, the host cells can be mammalian cells, yeast cells, insect cells or bacteria cells.

Methods of production, isolation and purification of the Lgs proteins, derivatives and analogs, e.g. by recombinant means, are also provided. In a specific embodiment, the invention concerns an isolated Lgs polypeptide or a fragment thereof, comprising an amino acid sequence of at least 80%, preferably at least about 85% sequence identity, more preferably at least 90% sequence identity, even more preferably at least 95% sequence identity, yet most preferably 100% identity with the sequence of amino acid residues 1 to 1464 of *Drosophila* Lgs of Figure 2 or amino acids residues of hLgs-1 of Figure 10.

In yet another embodiment the invention relates to chimeric proteins comprising a Lgs polypeptide fused to a heterologuos polypeptide or amino acid sequence. An example of such chimeric molecule comprises a Lgs polypeptide fused to an epitope tag sequence, glutathione-S-transferase protein or to a protein with an enzymatic activity, such as beta-galactosidase or alkaline phosphatase.

A further aspect of the invention concerns an isolated full length Lgs polypeptide, comprising the sequence of amino acid residues 1 to 1464 of Figure 2, or any Lgs polypeptide or fragment thereof comprised in this invention sufficient to provide a binding site for an anti-Lgs antibody.

In another embodiment the invention provides antibodies, which specifically recognize Lgs polypeptides. The antibodies can be a polyclonal or a monoclonal preparation or fragments thereof.

The present invention also provides an antisense oligonucleotide having a sequence capable of specifically hybridizing to RNA encoding vertebrate and invertebrate Lgs, so as to prevent translation of such RNA. This

invention further provides an antisense oligonucleotide having a sequence capable of specifically hybridizing to genomic DNA encoding a vertebrate and invertebrate Lgs, so as to prevent transcription of such genomic DNA. In one embodiment, the oligonucleotide comprises chemically modified nucleotides or nucleotide analogues.

The invention also relates to transgenic animals, e.g. *Drosophila*, mice, rats, chicken, frogs, pigs or sheep, having a transgene, e.g., animals that include and preferably express, a heterologous form of the Lgs genes described herein, or that misexpress an endogenous *lgs* gene. Such a transgenic animal can serve as a model to study diseases with disrupted Wnt signaling pathway, for the production of Lgs proteins, or for drug screening.

In yet another embodiment, the invention also features animals, e.g. *Drosophila*, mice, rats, chicken, frogs, pigs or sheep, having a mutation in a *lgs* gene, e.g. deletions, point mutations, foreign DNA insertions or inversions. Such animals can serve to study diseases characterized by disrupted Wnt function or in drug screening.

The invention also relates to therapeutic and diagnostic methods and compositions based on Lgs proteins and their homologues as well as the respective nucleic acids or fragments thereof. In particular, the invention provides for treatment of disorders of cell fate, differentiation or proliferation involving the Wnt pathway by administration of a therapeutic compound of the invention. Such therapeutic compounds include: *Drosophila* and vertebrate Lgs protein homologues or fragments thereof, antibodies or antibody fragments thereto, *lgs* antisense DNA or RNA, *lgs* double stranded RNA, and any chemical or natural occurring compound interfering with Lgs function, synthesis or degradation. In a preferred embodiment, a therapeutic product according to the invention is administered to treat a cancerous condition

or to prevent progression from a pre-neoplastic or non-malignant condition to a neoplastic or malignant state.

In a specific embodiment, a therapeutic product of the invention is administered to treat a blood disease or to promote tissue regeneration and repair. Disorders of cell fate, especially hyperproliferative or hypoproliferative disorders, involving aberrant or undesirable expression, or localization, or activity of the Lgs protein can be diagnosed by detecting such levels.

The present invention also provides a pharmaceutical composition comprising (a) an amount of a Lgs oligonucleotide in accordance with this invention capable of passing through a cell membrane and effective to reduce expression of Lgs and (b) a pharmaceutically acceptable carrier capable of passing through the cell membrane or to make the cell membrane permeable for such an oligonucleotide.

In yet another embodiment, the oligonucleotide is coupled to a moiety, which inactivates Lgs mRNA. In a specific embodiment, the moiety inactivating mRNA is a ribozyme. In another embodiment, the pharmaceutically acceptable carrier comprises a structure, which binds to a receptor on a cell capable of being taken up by the cells after binding to the structure.

In yet another embodiment the oligonucleotide is a double stranded lgs RNA molecule. Such ribonucleic acid fragments are about 18 to about 1000 nucleotides in length, preferably from about 20 to about 500 nucleotides in length, more preferably from 20 to 50, most preferably from 20 to 22 nucleotides in length and can be derived from the nucleotides sequences shown in Figure 2, 8 or 10.

Methods of preparing and employing antisense oligonucleotides, double stranded RNA oligonucleotides,

antibodies, nucleic acid probes and transgenic animals directed to Lgs are well known by persons skilled in the art.

The invention also includes methods of screening a plurality of chemical compounds to identify a compound, which specifically inhibits binding of mammalian Lgs proteins to β-Cat, Dorl (US provisional application No. 60/277,976) or any interacting partner identified by methods described by the invention. These methods comprise determining whether the binding of Lgs to an interacting partner is reduced in the presence of the compound, relative to the binding in the absence of the compound.

The invention also relates to nucleotide sequences and the respective peptides derived thereof comprising at least one of the homology domains between Drosophila and human Lgs described in Figure 7 and the use of said peptides to block Lgs function in cancer cells. Furthermore, the present invention comprises specific compounds that bind to said domains.

Brief description of the drawings

Figure 1 **(A)** Scanning electron micrographs of a wild type eye (left), a *sevenless-wingless* transgenic eye (centre), and an eye carrying the same transgene plus a loss of function *lgs* allele. Note the restoration of the hexagonal array of the ommatidia by mutant Lgs.
(B) Typical phenotype of animals with two mutated *lgs* alleles. The picture shows a pharate removed from the pupal case. Note the almost complete absence of legs, the wing to notum transformation (on the left side), and the complete lack of antennae.
(C) Intensification of the *wingless* lack of function phenotype by additional reduction of *lgs* function. These flies display notches in the wing margins (left panel), and dorsalization of ventral leg structures (right panel).

Figure 2 The *Drosophila lgs* sequence. cDNA is shown with introns from flies genomic DNA, introns are underlined. The first in-frame stop codon upstream of the ORF is underlined, the Kozak/Cavener sequence upstream of the initiator codon is marked by a bold underline, the beginning of the *poly(A) tail* is italicised.

Figure 3 **(A)** *Lgs* mRNA *in situ* hybridization. *Lgs* is maternally contributed and strongly and ubiquitously transcribed throughout embryonic development. The sense control probe reveals weaker transcription in a specific CNS pattern, probably due to repetitive elements transcribed in the opposite direction.
(B) Lgs-HA localization in peripodial membrane cells. Imaginal discs from larvae of genotype tub:*lgs*-HA were immunostained with mouse-anti-HA antibody and anti-mouse-FITC antibody conjugate. Lgs-HA specific staining can be seen in the nucleus (left panel). As a comparison, nuclei

are specifically stained with green YO-PRO fluorescent dye (Molecular Probes) (right panel). As a background control, imaginal discs not expressing HA-tagged Lgs protein were stained in the same way (data not shown). Similar results were obtained when the anti-dLgs antibody provided by this invention was used instead of the anti-HA.

Figure 4 Embryonic *lgs* mutant phenotype and epistasis analysis. ***Top***. Cuticle preparation of larvae derived from wild type (left), and $dLgs^{17E}/dLgs^{17E}$ mutant embryo (right). The ventral epidermis of wild type larvae displays regular denticle belts, spaced by naked cuticle. No naked cuticle is observed in $dLgs^{17E}/dLgs^{17E}$ mutant animals. ***Bottom***. The Wg signaling pathway was activated by ubiquitous expression of a constitutively active form of Arm (ΔArm) under the control of a hs-GAL4 driver. In these mutants embryo, ventral denticles are replaced by naked cuticle (left). Mutation of dLgs blocks over-activation of the Wg signaling pathway by ΔArm (right) and the phenotype is more reminiscent of *wg* loss of function mutations.

Figure 5 **(A)** Localization of Lgs protein in the absence and presence of NLS-Arm. HEK 293 cells were seeded into polylysine-coated 8 well chambers (Nalge-Nunc Internat.) and grown overnight at 37°C. Cells were then transfected by the lipofection method described below either with a green fluorescence tagged dLgs mammalian expression plasmid alone or together with a mammalian expression plasmid encoding for a nuclear localization sequence tagged Arm protein. The cells were then washed and fixed with 3.7% formaldehyde in PBS for 10 min. The washing step was repeated three times for 5 min before applying coverslips using Vectashield® mounting medium (Vector Laboratories, Inc.). **(B)** Co-immunoprecipitation of Lgs protein with Arm. HEK 293 cells at 50% confluence were transfected by a lipofection method. Seven µg of DNA were

diluted into 0.8 ml of OPTI-MEM Medium (Life Technologies, Inc.) and combined with 20 µl of Lipofectamine (Life Technologies, Inc.) in 0.8 ml OPTI-MEM. After incubation for 20 min, 1.6 ml of OPTI-MEM was added and the mixtures were overlaid onto monolayers of cells. After culturing at 37°C/5% CO_2 for 6 hr, 3 ml of OPTI-MEM containing 20% fetal calf serum (FCS) was added to the cultures. Cells were lysed 25 h after transfection in co-IP buffer (20 mM Tris HCl pH 7.5, 140 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1% Triton-X100, 10% glycerol, 1 mM Natrium vanadate, 50 mM NaF, and protease inhibitors). Immunoprecipitations were performed in co-IP buffer either using the rat IgG_1 anti-HA monoclonal antibody or the mouse anti-myc monoclonal antibody (Clone 9E10, Calbiochem) conjugated to protein G-agarose (Boehringer Mannheim). Control Immunoprecipitations were performed using rat or mouse IgG (Sigma-Aldrich). After 3h incubation at 4°C, beads were washed 4 times in washing buffer (20 mM Tris HCl pH 7.5, 140 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1% Triton-X100, 1 mM Natrium vanadate, 50 mM NaF) and resuspended in 25 µl of Laemmli buffer (Sambrook, Fritsch et al. 1989). Immune complexes were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) immunoblot assay using anti-GFP monoclonal antibody (Clontech Laboratories Inc.), followed by horseradish peroxidase conjugated secondary antibody (Amersham Pharmacia Biotech). Detection was performed using an enhanced chemiluminescence detection method (ECL, Amersham Pharmacia Biotech).

(C) Summary of the binding interactions in the yeast-two-hybrid assay. The desired cDNA were subcloned into the pLexA DNA binding domain vector (Clontech) and the pGJ4-5 activation domain vector (Clontech, sold as pAD). Yeast two hybrid was done by standard methods (Fields and Sternglanz 1994). Briefly, the appropriate pairs of plasmids were transformed together with the reporter plasmid pSH18-34 (Clontech) into the yeast strain EGY48 by the lithium acetate-polyethylene glycol method and

grown on selective media. Transformants were analyzed for beta galactosidase activity as described (Fields and Sternglanz 1994). To establish reproducibility the interactions were tested in both directions.

(D-E) *In Vitro* Binding Assays. Proteins were *in vitro* translated (IVT) using reticulocyte lysates (TNT-lysates, Promega Corporation) containing [^{35}S]-methionine (Amersham Pharmacia Biotech). Glutathione S-transferase (GST) fusion proteins were immobilized on glutathione-Sepharose and blocked in binding buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$, 10 % glycerol, 0.5% NP40, 0.05% BSA, and proteinase inhibitors) for 45 min. Two µg of immobilized GST proteins were then incubated for 1.5 hrs with 0.5-4 µl of IVT proteins in binding buffer. The beads were washed four times in washing buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$, 0.5% NP40) and boiled in Laemmli SDS sample buffer. The use of equivalent amounts of intact GST fusion proteins and successful IVT was confirmed by SDS-PAGE analysis using Coomassie staining or autoradiography, respectively.

(D) Binding of *in vitro* translated (IVT) Arm to GST-dLgs(1-732), GST-dLgs(733-1464) or GST alone (left panel), and of IVT Lgs to GST-Arm or GST alone (right panel).

(E) Binding of *in vitro* translated mouse ß-Cat to GST-hLgs(1-732) or GST alone.

Figure 6 Effect of Lgs on Tcf-Arm mediated transactivation of a Tcf response element driving a luciferase reporter gene. HEK293 cells at 50% confluence were transfected by a lipofection method. 450 ng of TOPFLASH luciferase reporter plasmid (Upstate biotechnology, New York, USA), 8 ng of pEGFP-Arm, 30-200 ng of pcDNA3-dLgs and 20 ng of a renilla luciferase reporter plasmid pRL-SV40 (Promega Corporation, Madison USA) to normalize the transfection efficiency, were diluted into 50 µl of OPTI-MEM Medium (Life Technologies,

Inc.) and combined with 2.4 µl of Lipofectamine (Life Technologies, Inc.) in 50 µl OPTI-MEM. After incubation for 20 min, 0.35 ml of OPTI-MEM was added and the mixtures were overlaid onto monolayers of cells. After culturing at 37°C/5% CO_2 for 6 hr, 0.45 ml of OPTI-MEM containing 20% FCS was added to the cultures. Cell extracts were prepared 48h after transfection and assayed for firefly and renilla luciferase activity as described by the manufacturer (Dual luciferase reporter assay system, Promega Corporation). All transfection experiments were carried out in triplicate, repeated at least three times, and normalized for renilla luciferase activity. Similar results are obtained using β-Cat and hLgs instead of Arm and dLgs, respectively.

Figure 7 (A) Distribution of short local alignments (sequence homology domains) between dLgs and hLgs/Bcl9. The number of each alignment refers to figure 7B which displays them in detail. A similar degree of homology is obtained by comparing homologues domains of dLgs and the predicted amino acid sequence of hLgs-1. hLgs/Bcl9 and hLgs display up to 95% homology in the same domains,
(B) Local alignments of dLgs with hLgs/Bcl9. A WWW server implementation of LALIGN (version 2.0u63 was used (matrix: pam120; gap penalties: -14/-4; alignment 4 edited by hand).

Figure 8 The human lgs/*bcl9* sequence.
(A) cDNA sequence.
(B) Protein sequence.

Figure 9 Prediction of the formation of coiled-coil structures by wild type dLgs, 4 mutant dLgs forms, and hLgs/Bcl9. One occurrence of a coiled coil between amino acids 526-539 is predicted for dLgs, and the overall picture is somewhat similar for hLgs/Bcl9. The peak is lost in $dLGS^{17E}$ with the single amino acids exchange at position 531, and it is cut off by premature termination

in the case of dLGS^{20F}. dLGS^{17P} with an amino acids exchange at position 532 has a reduced score, and the homozygous viable allele d*lgs*^{21L} with an amino acid exchange at position 509 is unaffected. A WWW server implementation of COILS version 2.1 was used with the MTDK matrix and without weights (Lupas, Van Dyke et al. 1991; Lupas 1997). All major peaks represent results obtained with a 14-residue window, the main peak also scores weakly with a 21-residue window, but nothing is detected with a 28-residue window. Remarkably, these mutated amino acids disrupting dLgs function are conserved in hLgs/Bcl9 (Figure 7).

Figure 10 (A) Putative *hLgs/bcl9* homologue (*hlgs-1*) partial C-terminal cDNA. Found by Blast search against hLgs/Bcl9 protein sequence. Following hs_genome/GS_mRNA was found which contains part of the hLgs-1 cDNA sequence: lcl|Hs11_9491_24_72_2. Most of the N-terminal region can be derived e.g. from following EST: BF752124, D63746, BG116685, and the hs_genome/GS_mRNA: lcl|Hs11_9491_22_28_8 (amino acid 1-225) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi)
(B) Predicted protein (C-terminal part of hLgs-1) (fragment) derived by translation of the predicted cDNA in (A). The N-terminus can to be derived by translation of the EST described above. The proteins contain all lgs sequence homology domains described in Figure 7.

Figure 11 Rescue experiments with hlgs/bcl9 in Drosophila. A tub:hlgs/bcl9 transgene was introduced into mutant dlgs20F/dlgs20F and dlgs17E/dlgs21L. These mutant flies are characterized by larval or pupal lethality. Pupae lack antenna and legs and have small wings **(A)**. In contrast, flies carrying the tub:hlgs/bcl9 transgene survive to adulthood and look like Lgs wild type flies **(B)**. This demonstrated that hLgs can replace dLgs function in *Drosophila*.

Figure 12 *In Vitro* Binding Assays, fine mapping. Proteins were *in vitro* translated (IVT) using reticulocyte lysates (TNT-lysates, Promega Corporation) containing [^{35}S]-methionine (Amersham Pharmacia Biotech). Glutathione S-transferase (GST) fusion proteins were immobilized on glutathione-Sepharose and blocked in binding buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$, 10 % glycerol, 0.5% NP40, 0.05% BSA, and proteinase inhibitors) for 45 min. Two µg of immobilized GST proteins were then incubated for 1.5 hrs with 0.5-4 µl of IVT proteins in binding buffer. The beads were washed four times in washing buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$, 0.5% NP40) and boiled in Laemmli SDS sample buffer. The use of equivalent amounts of intact GST fusion proteins and successful IVT was confirmed by SDS-PAGE analysis using Coomassie staining or autoradiography, respectively. **(A)** Binding of IVT dLgs fragments to GST-Arm (top), and of IVT ΔArm to GST-dLgs-fragments (bottom). **(B)** Precise mapping of the Arm binding sites in dLgs (top), and of the β-Cat binding sites in hLgs (bottom). The figures depict the binding of in vitro translated dLgs and hLgs fragments to GST-Arm and GST-β-Cat, respectively. The minimal protein fragment, which still binds to Arm or β-Cat comprises the dLgs-hLgs sequence homology domain 2 of figure 7. **(C)** Precise mapping of the Lgs binding sites in Arm. In vitro translated Arm fragment were tested for their binding to GST-dLgs(1-732)

Figure 13 Binding of mutants dLgs and hLgs to Arm/β-Cat. **(A)** Co-immunoprecipitation of mutant HA-dLgs-17E protein with GFP fused-Arm, -dTip, -dAPC and -Shaggy . HEK293 cells at 50% confluence were transfected by a lipofection method. Seven µg of DNA were diluted into 0.8 ml of OPTI-MEM Medium (Life Technologies, Inc.) and combined with 20 µl of Lipofectamine (Life Technologies, Inc.) in 0.8 ml

OPTI-MEM. After incubation for 20 min, 1.6 ml of OPTI-MEM was added and the mixtures were overlaid onto monolayers of cells. After culturing at 37°C/5% CO_2 for 6 hr, 3 ml of OPTI-MEM containing 20% fetal calf serum (FCS) was added to the cultures. Cells were lysed 25 h after transfection in co-IP buffer (20 mM Tris HCl pH 7.5, 140 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1% Triton-X100, 10% glycerol, 1 mM Natrium vanadate, 50 mM NaF, and protease inhibitors). Immunoprecipitations were performed in co-IP buffer either using the rat IgG_1 anti-HA monoclonal antibody or the mouse anti-myc monoclonal antibody (Clone 9E10, Calbiochem) conjugated to protein G-agarose (Boehringer Mannheim). Control Immunoprecipitations were performed using rat or mouse IgG (Sigma-Aldrich). After 3h incubation at 4°C, beads were washed 4 times in washing buffer (20 mM Tris HCl pH 7.5, 140 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1% Triton-X100, 1 mM Natrium vanadate, 50 mM NaF) and resuspended in 25 µl of Laemmli buffer. Immune complexes were analyzed by SDS-PAGE/immunoblot assay using anti-GFP monoclonal antibody (Clontech Laboratories Inc.), followed by horseradish peroxidase conjugated secondary antibody (Amersham Pharmacia Biotech). Detection was performed using an enhanced chemiluminescence detection method (ECL, Amersham Pharmacia Biotech). **(B-C)** *In Vitro* Binding Assays. Proteins were *in vitro* translated (IVT) using reticulocyte lysates (TNT-lysates, Promega Corporation) containing [^{35}S]-methionine. Glutathione S-transferase (GST) fusion proteins were immobilized on glutathione-Sepharose and blocked in binding buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$, 10 % glycerol, 0.5% NP40, 0.05% BSA, and proteinase inhibitors) for 45 min. Two µg of immobilized GST proteins were then incubated for 1.5 hrs with 0.5-4 µl of IVT proteins in binding buffer. The beads were washed four times in washing buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$, 0.5% NP40) and boiled in Laemmli SDS sample buffer. The use of

equivalent amounts of intact GST fusion proteins and successful IVT was confirmed by SDS-PAGE analysis using Coomassie staining or autoradiography, respectively. **(B)** Binding of IVT wild type dLgs and dLgs-17E mutant to GST-ΔArm or GST alone (left panel), and of IVT wild type dLgs(354-555) or dLgs(354-555)-17E and -17P mutants to GST-ΔArm or GST alone (right panel). **(C)** Binding of IVT wild type hLgs/Bcl9 to GST alone or GST-ß-Cat (top) and of IVT hLgs(Δ345-385) (also named hLgsdn) to GST alone or GST-ß-Cat (bottom). Mutations in the conserved amino acids of the sequence homology domain 2 of Figure 7 abolish binding of Lgs to Arm and β-Cat.

Figure 14 Down-regulation of dLgs protein levels by RNA interference. dLgs dsRNA was synthesized by PCR from pBS-dLgs (full length cDNA) using the T7 promoter containing dsRNA-Lgs-R1
(TAATACGACTCACTATAGGGAGACCACTTCCATGCTCATTTCGTCATTA) and dsRNA-Lgs-F1
(TAATACGACTCACTATAGGGAGACCACTAGGATCTCTCGACAACAATG)
primers. As a control a PCR fragment was amplified from Arm cDNA using following primers: F primer (TAATACGACTCACTATAGGGAGACCACACAAGACCAAGTGGACGATATG),
R Primer
(TAATACGACTCACTATAGGGAGACCACAATTTGCAAGCAATCTGTGAC).
The amplified 700 base pairs products were purified using the PCR-Purification kit from Quiagen and the DNA was eluted with 50 µl water. The DNA concentration was determined by UV absorbtion. The RNA synthesis reaction was then performed in 50µl volume with 1µg of the purified PCR products using the MEGAscript™ kits from Ambion. The DNA templates were removed with RNase-free DNAase and the dsRNAs were purified by phenol-chloroform extraction and ethanol precipitation. The RNAs became double-stranded during the synthesis reaction as confirmed by native agarose gel electrophoresis in TBE. For the RNA interference experiments, S2 cells were

propagated in Schneider S2 Drosophila medium (GIBCO) supplemented with 10% FCS. One day before transfection one million cells were seeded into 6 well plates and growth overnight at 25°C. A total of 5 ug DNA and dsRNA was complexed with 20 ul of CellFectine lipid mix (GIBCO) in 1.2 ml serum free growth medium (DES expression medium, Invitrogen, Carlsbad,USA). As a control, EGFP (Clontech Laboratories Inc., Palo Alto, USA) protein was expressed in the same cells under the control of the methallothionin promoter (vector used: pMT-V5/HISB, Invitrogen). The complexes were incubated for 15 minutes at RT and then added to the cells from which the normal growth medium was replaced with 1 ml serum free medium. Four hour later 1.2 ml growth medium supplemented with 30%FCS was added to the cells. One day after transfection the medium was replaced with fresh medium with 10% FCS. Where an expression plasmid under the control of the insect metallothionin promoter (pMT/V5-HisB, Invitrogen) was transfected together with the dsRNA, copper sulfate was added to the cells to a final concentration of 0.5 mM. Cells were lysed in RIPA buffer 2 days after transfection. The cleared lysates were analyzed by SDS-PAGE/immunoblot assay using anti-Lgs polyclonal antiserum described herein and anti-GFP monoclonal antibody (Clontech Laboratories Inc.), followed by horseradish peroxidase conjugated secondary antibody (Amersham Pharmacia Biotech). Detection was performed using an enhanced chemiluminescence detection method (ECL, Amersham Pharmacia Biotech). **Top panel** Downregulation of endogeneous Lgs expression by Lgs dsRNA. As a control, cells were treated with Arm dsRNA. EGFP expression is not affected by the treatment with lgs dsRNA. **Lower panel** Downregulation of exogeneous dLgs expression. dLgs levels are brought under endogeneous levels by Lgs dsRNA treatment.

Figure 15 (A) Effect of the expression of Lgs sequence homology 2-peptides on Tcf transcriptional activity. HEK293 cells at 50% confluence were plated into 24-well

plates and transfected by a lipofection method. 240 ng of TOPFLASH luciferase reporter plasmid (Upstate biotechnology, New York, USA), 4 ng of pcDNA3-ΔArm, 200 ng of pcDNA3-EGFP-Lgs-peptide and 10 ng of a renilla luciferase reporter plasmid pRL-SV40 (Promega Corporation, Madison USA) were diluted into 25 µl of OPTI-MEM Medium (Life Technologies, Inc.) and combined with 1.2 µl of Lipofectamine (Life Technologies, Inc.) in 25 µl OPTI-MEM. After incubation for 20 min, 0.175 ml of OPTI-MEM was added and the mixtures were overlaid onto monolayers of cells. After culturing at 37°C/5% CO_2 for 6 hr, 0.225 ml of OPTI-MEM containing 20% FCS was added to the cultures. Cell extracts were prepared 48h after transfection and assayed for firefly and renilla luciferase activity as described by the manufacturer (Dual luciferase reporter assay system, Promega Corporation). All transfection experiments were carried out in triplicate, repeated at least three times, and normalized for renilla luciferase activity. **(B)** Effect of Lgs HD2 peptides on Tcf-driven luciferase activity in SW480 colon carcinoma cells (American Tissue Culture Collection, ATCC). In these cancer cells the Wnt pathway is constitutively active due to a mutation in the APC tumor suppressor gene. As a positive control, a dominant negative hTcf4 (dnTcf4) protein was used (Roose, Huls et al. 1999). Cells were transfected as described above but using Lipofectamine 2000 (GIBCO Life Technologies) instead of Lipofectamine following the manufacturer recommendations.

Detailed description of the invention

The Wnt signaling cascade is essential for the development of both invertebrates and vertebrates, and has been implicated in tumorogenesis. The *Drosophila wg* genes are one of the best characterized within the Wnt-

protein family, which includes more than hundred genes. In the Drosophila embryo, *wg* is required for formation of parasegment boundaries and for maintenance of *engrailed* (*en*) expression in adjacent cells. The epidermis of embryo defective in *wg* function shows only a rudimentary segmentation, which is reflected in an abnormal cuticle pattern. While the ventral cuticle of wild type larvae displays denticle belts alternating with naked regions, the cuticle of wg mutant larvae is completely covered with denticles. During imaginal disc development, *wg* controls dorso-ventral positional information. In the Leg disc, *wg* patters the future leg by the induction of ventral fate (Struhl and Basler 1993). In animals with reduced *wg* activity, the ventral half of the leg develops into a mirror image of the dorsal side (Baker 1988). Accordingly, reduced wg activity leads to the transformation of wing to notal tissue, hence the name of the gene (Sharma and Chopra 1976). In the eye disc, *wg* suppresses ommatidial differentiation in favor of head cuticle development, and is involved in establishing the dorso-ventral axis across the eye field (Heberlein, Borod et al. 1998).

Additional genes have been implicated in the secretion, reception or interpretation of the Wg signaling. For instance, genetic studies in *Drosophila* revealed the involvement of *frizzled* (*Dfz*), *dishevelled* (*dsh*), *shaggy/zeste-white-3* (*sgg/zw-3*), *armadillo* (*a r m*), *adenomatous polyposis coli* (*apc*), *axin*, and *pangolin (pan)* in wg signaling. The genetic order of these transducers has been established in which Wg acts through Dsh to inhibit Sgg, thus relieving the repression of Arm by Sgg, resulting in the cytoplasmic accumulation of Arm and its translocation to the nucleus. In the nucleus Arm interacts with Pan to activate transcription of target genes. Vertebrate homologues have been identified for all these components (for an updated review see (Peifer and Polakis 2000)), suggesting that novel identified members

of the Drosophila signaling pathway may likely have vertebrate counterparts.

Mutations leading to nuclear accumulation of the mammalian homologue of Arm, ß-Cat, and consequently to constitutive activation of the Wnt pathway have been observed in many type of cancers, including colon, breast, skin, tyroid, medulloblastoma, and head and neck cancer (Morin 1999; Polakis, Hart et al. 1999). Currently, there are no known therapeutic agents effectively inhibiting ß-Cat transcriptional over-activation in these cancers. This is partly due to the fact that many of the essential components required for ß-Cat full activation, nuclear translocation and for its role in transcription of target genes are still unknown.

In order to identify new positive acting components of Wg signaling pathway Drosophila genetic was used. Methods to generate a particular genetically modified Drosophila strain and how to screen for specific mutations in a define signaling pathway are well known by people skilled in the art and are not part of this invention.
dLgs was found in a genetic screen for dominant suppressors of the rough eye phenotype induced by a transgene which drives ectopic *wg* expression under the control of the *sevenless* (*sev*) promoter during eye development in Drosophila (Brunner 1997) (Figure 1). d*Lgs* mRNA is maternally contributed and strongly and ubiquitously expressed during all the developmental stages (Figure 3A). Consequently, embryos lacking both embryonal and maternal *dlgs* are characterized by a strong segment polarity phenotype, while weaker loss of function *dlgs* mutants display pupal lethality with transformation of sternites to pleura and a partial or complete loss of the antennae and the legs (hence its name). The wings of these animals are usually not affected, but are occasionally transformed into secondary notum (Figure 1). The fact that similar phenotypes are caused by loss of

function of *wg*, *dsh* and *arm* confirmed the essential role of *lgs* in the Wg signaling pathway.

dLgs is located on the fourth chromosome. The *dlgs* gene was cloned by positional cloning and genomic walk, techniques frequently used by persons skilled in the art. Dlgs encodes for a 1464 amino acid protein of an expected molecular mass of 153 kDa. The dLgs protein is predicted to be predominantly hydrophilic and positively charged with a small hydrophobic stretch around amino acid 300 (Figure 2). Neither obvious dLgs homologue nor any characterized functional motif can be found by common search tools (http://dot.imgen.bcm.tmc.edu:9331). However, by modification of the standard search parameters, several short stretches of amino acids within the dLgs protein are found to be highly homologue to a human protein, known as Bcl9, which has been linked to the development of B-cell lymphoma (Willis, Zalcberg et al. 1998; Busson-Le Coniat, Salomon-Nguyen et al. 1999), and to several translated EST coming from a predicted gene on chromosom 11. Interestingly, Bcl9, from now on named hLgs/Blcl9, displays similar structural feature compared to dLgs, like length, hydrophility and the presence of a predicted coiled region (Figure 9). In addition, it is remarkable that the short stretches of homology occur in a similar spacing and in the same succession as in dLgs (Figure 7). As we show below, despite the overall very modest homology, hLgs/Bcl9 revealed to be the true functional human homologue of dLgs, and its function, as well as any lgs homologues, is hence part of the present invention.

In order to gain further biochemical and functional insight into the role of Lgs in the Wg/Wnt signaling pathway, we screened for potential interaction partners. The data presented herein (see examples) show that dLgs but not loss of function dLgs mutants, physically interacts with Arm and Doll (For Doll interaction see US provisional application No. 60/277,976). In contrast, no

interaction can be detected with other Wg pathway components such as dAPC and Shaggy. Accordingly, epistasis experiments in Drosophila embryo clearly place dLgs at the same level or downstream of Arm (Figure 4).

The interaction with Arm is also confirmed in mammalian cells, where dLgs can be directed to the nucleus in the presence of nuclear but not cytoplasmic Arm (Figure 5A). Moreover, when co-transfected with Arm, Lgs increases the transcriptional activity of hTcf (see examples and Figure 6). Similarly, we report herein the binding of hLgs/Bcl9 to ß-Cat and its effect on ß-Cat dependent transcriptional activation. We also demonstrate that dLgs and hLgs bind Arm and β-Cat, respectively, with the homology region No. 2 described in figure 7, and that the homology region No. 1 is also essential for Lgs function (since it binds to Dsh, another essential component of the Wg/Wnt pathway (provisional patent application No. 60/221,502).

Methods and vectors to achieve such results are well known in the art, and are reported herein by mean of examples.

In summary, the biochemical interactions demonstrated herein between dLgs and Arm and between their human homologues hLgs/Bcl9 and ß-Cat, respectively, in conjunction with a Tcf-activation assay, complement genetic studies in *Drosophila* and indicate that Lgs proteins are positive regulators of the Wg/Wnt signaling pathway and are required for β-Cat dependent gene activation. Importantly, since Lgs is involved in late events of the Wg/Wnt signaling cascade, blocking its function, e.g. by interfering with its interaction with β-Cat or Dsh, would result in blockade of the Wnt signal propagation, also where β-Cat is out of control due to oncogenic mutations in such a pathway. Consequently, this invention also relates to therapeutic and diagnostic methods and compositions based on Lgs proteins and their

homologues as well as the respective nucleic acids or fragments thereof. In particular, the invention provides for treatment of disorders of cell fate, differentiation or proliferation involving the Wnt pathway by administration of a therapeutic compound of the invention. Such therapeutic compounds include but are not limited to: *Drosophila* and vertebrate Lgs protein homologues or fragments thereof, antibodies or antibody fragments thereto, *lgs* antisense DNA or RNA, *lgs* double stranded RNA, and any chemical or natural occurring compound interfering with Lgs function, synthesis or degradation.

The invention also includes methods of screening a plurality of chemical compounds to identify a compound, which specifically inhibits binding of mammalian Lgs proteins to β-Cat, Doll (US provisional application No. 60/277,976) or any positive acting, interacting partner identified by methods described by the invention. These methods comprise, but they are not limited to, determining whether the binding of Lgs to an interacting partner is reduced in the presence of the compound, relative to the binding in the absence of the compound. Such assays can be performed *in vivo* or *in vitro*. If an *in vivo* assay is used, then the interacting proteins have to be fused e.g. to a protein which allow the detection of such interaction. Such an example are mammalian or yeast two hybrid assays or methods measuring the energy transfer between a donor and an acceptor prctein *in vivo*. If an *in vitro* assay is to be used, several methods are available to persons skilled in the art. These include but are not limited to fluorescent resonance energy transfer based methods (Kolb, Burke et al. 1997; Sittampalam, Kahl et al. 1997). All the methods indicated in this context are well known by people skilled in the art.

The invention also relates to Lgs nucleotide sequences and the respective peptides derived thereof comprising at least one of the homology domains between Drosophila and human Lgs described in Figure 7 and the use of said peptides to block Lgs function in cancer cells. Suitable techniques are known in the art for administering peptide to tumors. This can be achieved by direct administration of the peptide itself together with an appropriate pharmaceutical preparation which allow the penetration of such peptides into cells, or by mean of a gene therapy format. The latter bases of the administration of a DNA sequence coding for the peptide using suitable expression vectors. Such vectors are known in the art and it is in the skill of the artisan to select an appropriate one. In the tumor cells, the peptides will bind to their interaction partner, e.g. β-Cat if the homology domain 2 peptide is chosen, and titrate it away from the endogeneous Lgs proteins thus preventing expression of target genes by uncontrolled β-Cat.

The above disclosure generally describes the present invention. A more complete understanding can be achieved by the following specific examples, which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

1. Definitions

The terms "Lgs sequence", "Lgs polypeptide", "Lgs protein" when used herein encompasses native *vertebrate and invertebrate* Lgs and Lgs variant sequences (which are further defined herein).

A "wild type Lgs sequence" comprises a polypeptide having the same amino acid sequence as a Lgs protein derived from nature. Such wild type sequence of Lgs can be isolated from nature or produced by recombinant and/or

synthetic means. The term "wild type sequence Lgs" specifically encompasses naturally occurring truncated forms, naturally occurring variant forms (e.g., alternatively spliced forms) and naturally occurring allelic variants of Lgs. In one embodiment of the invention, the native Lgs sequence is a mature or full-length Lgs sequence comprising amino acids 1 to 1464 of Figure 2 or amino acids 1 to 1394 of Figure 8.

"Lgs variant" means an active Lgs, having at least about 50% amino acid sequence identity with the amino acid sequence of residue 1 to 1464 of the *Drosophila* Lgs polypeptide of the sequence of Figure 2 or amino acids 1 to 1394 of Figure 8. The term „lgs variant" however, does also include functional homologues of Lgs in the Wnt pathway.

"Percent (%) amino acid sequence identity" with respect to the Lgs sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that is identical with the amino acid residues in the Lgs sequence described herein, after aligning the sequence and introducing gaps, if necessary, to achieve the maximum percentage sequence identity, and not considering any conservative amino acid substitution as part of the sequence identity. The % identity values used herein can be generated by WU-BLAST-2, which was obtained from (Tatusova TA 1999). WU-BLAST-2 uses several search parameters, most of which are set to the default values.

The term "positive", in the context of sequence comparison performed as described above, includes residues in the sequence compared that are not identical but have similar properties (e.g. as a result of a conservative substitution). The % value of positive is determined by the fraction of residues scoring a positive value in the BLOSUM 62 matrix divided by the total number of residues in the longer sequence as defined above.

In a similar manner, "percent (%) nucleic acid sequence identity" with respect to the coding sequence of the Lgs polypeptides identified herein is defined as the percentage of nucleotide residues in a candidate sequence that are identical with the nucleotide residues in the Lgs coding sequence. The identity values used herein can be generated using BLAST module of WU-BLAST-2 set to the default parameters.

The term "epitope tag" refers to a chimeric polypeptide comprising a Lgs polypeptide fused to a „tag polypeptide". The tag polypeptide has enough residues to provide an epitope against which an antibody can be made, yet is short enough that it does not interfere with the activity of the Lgs polypeptide to which it is fused.

Nucleic acids are "operably linked" when they are placed in a functional relationship with another nucleic acid sequence.

The term „epistasis" means hierarchy in gene action. Epistasis experiments are performed to place components of a signaling pathway in the right order.

The term „rescue experiments" are designed to determine which gene is responsible for a specific mutant phenotype. Specifically, mutant embryos are injected with coding or genomic DNA, and the effect of the introduced DNA is determined on the basis of the capacity to revert the mutant phenotype.

„Active" or „activity" refers to forms of Lgs polypeptides that retain the biological and/or immunological activity. A preferred activity includes for instance the ability to positively modulate the Wnt signaling pathway.

The term "antagonist" is used in a broad sense, and includes any molecule that partially or fully inhibits,

blocks or neutralizes a biological activity of Lgs polypeptides described herein. In a similar way, the term "agonist" is used in the broadest sense and includes any molecule that mimics a biological activity of an active Lgs polypeptide.

„Treatment" refers to both therapeutic treatments and prophylactic or preventive measures, wherein the objective is to prevent or slow down the targeted pathologic condition or disorder. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in whom the disorder is to be prevented.

2. General Methods

Example I: Isolation of *lgs* cDNA

a) Drosophila Lgs:
Lgs was found by positional cloning. A *Drosophila* genomic region of about 150 kDa was cloned, and by a combination of genetic and molecular methods known in the art, the region containing the gene was reduced to 75 kDa. *dLgs* was then identified by the analysis of mutant sequences and by rescue experiments. Alternatively, *lgs* primers can be used to screen cDNA libraries as described in (Sambrook, Fritsch et al. 1989).

b) Human Lgs:
Human Lgs was identified by searching a public sequence database (http://www.ch.embnet.org/software/aBLAST.html) with the amino acid sequence of *Drosophila* Lgs. DLgs shows statistically significant similarity to the human Bcl9 protein, a previously described protein of unknown

function. The main regions of homology are Lgs amino acids 323-554 and Bcl9 amino acids 177-383.
The hlgs/bcl9 full-length cDNA was assembled from partial EST clone sequences (NCBI:AI338959 and NCBI:AL039210) and PCR fragments obtained on human cDNA and genomic DNA preparations. After the assembly process, the sequence was verified by crosschecking with genomic DNA sequences and the publicly available data.

Example II: Use of *lgs* as a hybridization probe

The following method describes use of a non-repetitive nucleotide sequence of *lgs* as a hybridization probe. The method can be applied to screen for lgs homologues as well. DNA comprising the sequence of *lgs* (as shown in Figures 2, 8 and 10) is employed as probe to screen for homologue DNAs (such as those included in cDNA or genomic libraries).
Hybridization and washing of the filters containing either library DNAs is performed under standard high stringency conditions (Sambrook, Fritsch et al. 1989). Positive clones can be used to further screen larger cDNA library platings. Representative cDNA-clones are subsequently cloned into pBluescript (pBS, Stratagene) or similar cloning vectors, and sequenced.

Example III: Use of *lgs* as a hybridization probe for *in situ* hybridization.

In situ hybridization of Drosophila *lgs* mRNA can be performed in embryo as described in (Tautz and Pfeifle 1989). However, with small modifications it can also be used to detect any mRNA transcript in Drosophila larval imaginal discs or vertebrate tissue sections. Labeled RNA probes can be prepared from linearized *lgs* cDNA (as

showed in Figure 2), or a fragment thereof, using the DIG RNA labeling Kit (SP6/T7) (Boehringer Mannheim) following the manufacturer's recommendations. A similar method can be used with hLgs as a hybridization probe to screen human tissues.

Example IV: Expression of *lgs* in *Drosophila melanogaster*

Lgs can be expressed in Drosophila in the whole organism, in a specific organ or in a specific cell type, during the whole life or only at a specific developmental stage, and at different levels. An overview of the standard methods used in Drosophila genetics can be found in (Brand and Perrimon 1993; Perrimon 1998; Perrimon 1998).

Generation of lgs mutant embryos

Mosaic germlines are generated by help of site-specific recombination through the FLP recombinase (Xu and Rubin 1993). Females of the genotype *hsp70:flp,tub:>dlgs-cDNA>Gal4/+; dlgs20F/dlgs20F* (mutant dlgs alleles) are heat-shocked at 37°C for 1 hr during the third instar larval stage to induce FLP-directed recombination and later mated to *UAS:GFP/UAS:GFP; dlgs20F/yellow+* males. Germline mosaics are induced in homozygote d*lgs20F*-mutant females carrying one copy of a d*lgs* cDNA ("rescuing") transgene flanked by two recombination target sites (symbolized by ">") and followed by a Gal4 coding sequence. The source of recombinase is a first chromosome insertion of a fusion of the *hsp70* promoter (denoted by "*hsp70*") to the FLP coding sequence. Excision of the rescuing *dlgs* cDNA from cell clones in larval tissue gives rise to adult female germ lines that produce oocytes that do not contain neither zygotic nor maternally contributed information for the production of functional Lgs protein. At the same time the Gal4 coding sequence is spliced to the transgenic promoter sequence,

which induces formation of the heterologous transcriptional activator. Upon fertilization of the zygotically and maternally *dlgs* mutant oocytes, the Gal4 transcriptional activator turns on a UAS:GFP transgene contributed by the paternal sperm which mark the mutant eggs by GFP expression. With this method, about fifty percent of the produced eggs express GFP and thus have excised the *lgs* rescue transgene. For analysis, cuticles are prepared by standard techniques from mutant embryos, and examined by dark field microscopy.

Generation of dlgs mutant embryos expressing constitutively active Arm

In order to express constitutively active Arm ("ΔArm"), females of the genotype described above are heat shocked at 37°C for 1 hr during late pupal stages and mated to males of the genotype *UAS:GFP,UAS:ΔArm/UAS:GFP,UAS:ΔArm; dlgs^{20F}/yellow^{+}*. Due to the additional presence of the UAS:ΔArm transgene in these males all offspring that had arisen from a *dlgs* mutant oocytes expressed both the marker protein GFP and a constitutively active Arm protein that permanently induced Wg target genes.

Example V: Expression of Lgs in E. coli

The following method describes recombinant expression of Lgs in bacterial cells. Alternatively, recombinant proteins can be produced and isolated from insect and mammalian cels (Sambrook, Fritsch et al. 1989). DNA encoding full-length or a truncated Lgs form is fused downstream of an epitope tag or glutathione-S-transferase (GST) cDNA and a thrombin cleavage site contained within an inducible bacterial expression vector. Such epitope tags include poly-his, S-protein, thioredcxin, and immunoglobin tags. A variety of plasmids can be employed, including commercially available plasmid such as pGEX-4T (Pharmacia).

Briefly, a bacterial expression plasmid containing the Lgs sequence, for instance fused to a GST-sequence is transformed by conventional methods into protease deficient E.coli such as BL21 (e.g. Stratagene). A bacterial colony containing the plasmid is then expanded overnight in selection medium to reach saturation. The next morning, this culture is diluted 1:100 and bacterial are allowed to grow to an optical density (OD_{600}) of 0.6. Protein production is initiated by addition of an inducer of the promoter under which GST-Lgs fusion protein is expressed. A variety of inducers can be employed depending on the expression vector used, including IPTG.

Expressed GST tagged Lgs can then be purified, for instance, using affinity beads or affinity chromatography, such as glutathione beads (commercially available e.g. from Pharmacia). Extracts are prepared by lysing the Lgs-expressing bacteria in sonication buffer (10 mM Tris HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1.5% sarkosyl, 2% Triton-X-100, 1 mM DTT and protease inhibitors), followed by short sonication on ice (e.g. 3 times 20 seconds at middle power) and centrifugation. Cleared supernatants are then incubated under gentle rotation for example with glutathione beads for 2 hrs at 4°C. Next beads are washed several time in washing buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$, 0.5% NP40), and finally stored in storage buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM MgCl2, 10 % glycerol, 0.5% NP40, and proteinase inhibitors). Alternatively, a His-tagged or IgG tagged Lgs can be purified using Ni^{2+}-chelate affinity chromatography or Protein A or Protein G column chromatography, respectively.
The quality of the preparations can be checked e.g. by SDS-gel electrophoresis and silver staining or Western blot.

In case the epitope tagged has to be cleaved, several methods are available depending on the presence of a cleavage site between the epitope tagged and the Lgs protein. For example, it is possible to produce a GST-Lgs fusion protein containing a thrombin cleavage site right before the first Lgs amino acid. Briefly, a GST-Lgs preparation on glutathione-affinity beads is washed several times in cleavage buffer (50 mM Tris HCl pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT). Thrombin is then added and the samples are incubated for over 16 hrs at room temperature. Supernatants are then collected and analyzed for successful cleavage of Lgs from the beads by polyacrylamide gel electrophoresis and silver staining or Western blot. The purified proteins can be used e.g. to generate anti-Lgs antibodies as described in (Harlow and Lane 1988)

Example VI: Protein-protein interactions involving Lgs

An *in vitro* co-immunoprecipitation assay can be performed to find or confirm Lgs interaction partners. For instance, HEK293 cells at 50% confluence are transfected by a lipofection method. For this purpose, mammalian expression vectors containing cDNA encoding for tagged Lgs and potential interaction partners are combined with Lipofectamine transfection reagent (Life Technologies, Inc.) following the manufacturer recommendations, and overlaid onto monolayers of cells. Cells are lysed 25 hrs after transfection in co-IP buffer (20 mM Tris HCl pH 7.5, 140 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1% Triton-X100, 10 % glycerol, 1 mM Natrium vanadate, 50 mM NaF, and protease inhibitors). Immunoprecipitations are performed in co-IP buffer using anti-tag antibodies (e.g. anti-HA, clone 3F10, Boehringer Mannheim) conjugated to protein G-agarose (Boehringer Mannheim). Control immunoprecipitations are performed using rat or mouse IgG (Sigma-Aldrich). After 3 hrs incubation at 4 °C, beads are washed 4 times in washing

buffer (20 mM Tris HCl pH 7.5, 140 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1% Triton-X100, 1 mM Natrium vanadate, 50 mM NaF) and resuspended in 25 µl of Laemmli buffer. Immune complexes are analyzed by SDS-PAGE/immunoblot assay using anti-Lgs polyclonal antibodies provided by the invention or anti-tag antibodies, followed by horseradish peroxidase conjugated secondary antibody (Amersham Pharmacia Biotech). Detection can be performed using an enhanced chemiluminescence detection method (e.g. ECL, Amersham Pharmacia Biotech).

A GST-fusion protein *in vitro* binding assay can be performed e.g. to map binding domains, confirm an interaction partner or find additional interacting proteins. For this purpose, proteins are *in vitro* translated (IVT) using reticulocyte lysates (TNT-lysates, Promega Corporation) containing [^{35}S] methionine following the instructions provided by the manufacturer. Glutathione S-transferase (GST) fusion proteins, produced as illustrated in the Example V, are immobilized on glutathione-Sepharose and blocked in binding buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM MgCl2, 10% glycerol, 0.5% NP40, 0.05% BSA, and proteinase inhibitors) for 45 min. Two µg of immobilized GST proteins are then incubated for 1.5 hrs with 0.5-4 µl of IVT proteins in binding buffer. The beads are washed four times in washing buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$, 0.5% NP40) and boiled in Laemmli SDS sample buffer. The use of equivalent amounts of intact GST fusion proteins and successful IVT of the AR has to be confirmed by SDS-PAGE analysis using Coomassie staining or autoradiography, respectively.

A yeast two hybrid assay can additionally be performed to confirm the results of the *in vitro* binding assays described above or to screen a cDNA library for new interaction partners (Fields and Sternglanz 1994). To

confirm a specific binding (e.g. β-Cat) or to map the binding region between Lgs and an interaction partner the desired cDNAs are subcloned into appropriate yeast expression vectors that link them either to a Lex DNA binding domain (e.g. pLexA, Clontech) or an acidic activation domain (e.g. pGJ4-5, Clontech). The appropriate pair of plasmids is then transformed together with a reporter plasmid (e.g. pSH18-34, Clontech) into an appropriate yeast strain (e.g. EGY48) by the lithium acetate-polyethylene glycol method and grown on selective media (Sambrook, Fritsch et al. 1989). Transformants are analyzed for reporter gene activity as described by the manufacturer of the vector-reporter plasmid used. To establish reproducibility the interactions is tested in both directions.

To isolate novel Lgs-binding proteins (Bartel, Fields "The Yeast two-Hybrid System" Oxford UP, 1997) an appropriate yeast strain is transformed with a beta-Galactosidase reporter plasmid, a yeast expression vector containing Lgs cDNA, or parts thereof (such as the dLgs/hLgs-homology regions), fused to the LexA DNA-binding domain sequence ("bait vector") and a second yeast expression vector containing a transcriptional activation domain fused to a collection of cDNA sequences ("prey vector" library, e.g. RFLY1 0-12 h embryo library, described in PNAS 93, 3011ff.). The triple transformants containing the reporter plasmid, and the bait and prey vectors are then grown on selective media, and selected for interaction-dependent activation of the auxotrophic and beta-Galactosidase reporters. From selected clones the respective prey construct is reisolated and the specificity of bait/prey-interaction is assessed, by checking for absence of interaction with unrelated bait-constructs. Finally the confirmed interactors are sequenced and full-length cDNAs are assembled and tested again for specific interaction with the bait.

In two unrelated screens using Lgs full-length and Lgs N (amino acids 1-732) as baits, we isolated independent

cDNA clones of a novel protein, Daughter of Legless (dDoll). Doll specifically binds to the homology domain 1 of dLgs (amino acids 318-345) and hLgs/BCL9 (amino acids 177-205) through its C-terminal PHD-finger, a Zinc-finger related structural motif (see US provisional application No. 60/277,976).

Example VII: Immunohistochemistry

Localization of Lgs protein can be performed on *Drosophila* embryo, imaginal discs, adult tissue sections, vertebrate tumor cell lines, or vertebrate tissues using the anti-Lgs antibodies provided by this invention. For instance, if a transformed cell line like HEK 293 cells (ATCC) is used, cells are seeded into polylysine-coated 8 well chambers (Nalge-Nunc Internat.) and grown overnight at 37°C. The next day, cells are fixed with 3.7% formaldehyde in PBS for 10 min, permeabilized in 0.5% Triton-X-100 for another 10 min, and blocked with a 1:1000 dilution of pre-immunoserum in 2% BSA-PBS for 1 h at RT. Cells are then incubated with a 1:1000 dilution of anti-Lgs polyclonal rabbit immunoserum provided by this invention for 2 hrs at RT. The slides are washed three times for 5 min in PBS and incubated with a 1:200 dilution (v/v) of TRITC-conjugated swine anti-rabbit immunoglobulin (Dako, Inc.). The washing step is repeated before applying coverslips using Vectashield® mounting medium (Vector Laboratories, Inc.). Detection of other proteins such as Arm/β-Cat or Pan/Tcf can be performed in the same way using specific antibodies. As a positive control part of the cells can be transfected e.g. by a lipofection method with a Lgs expression plasmid, such as pcDNA3.1 (Invitrogen). Two days after transfection, control cells are stained with anti-Lgs antibodies as described above.

Example VIII: Luciferase reporter gene assays

The effect of Lgs on Tcf transactivation activity can be performed in a cell culture system using a Tcf reporter gene. A Tcf-responsive reporter gene is a contruct which comprises a readily detectable or assayable gene such β-galactosidase, green fluorescent protein, chloramphenicol acetyltransferase or luciferase, linked *in cis* to a Tcf response element and a minimal promoter. Depending on the expression vectors used, this protocol can be applied for mammalian as well as for *Drosophila* cell lines. For instance, HEK293 cells (ATCC) are a well suitable system. Hereby, Lgs and β-Cat full length cDNA are cloned into a mammalian expression vector, such as pcDNA3 (Invitrogen), and transfected together with a Tcf driven luciferase reporter plasmid (TOPFLASH, Upstate biotechnology, New York, USA) into HEK 293 cells. Any means for introducing genetic material into cells can be used, including but not limited to infection, electroporation or transfection. For instance, to introduce DNA into HEK 293 cells, a lipofection agent like the Lipofectamine transfection reagent (Life Technologies, Inc.) can be used. A renilla luciferase reporter plasmid, e.g. pRL-SV40, (Promega Corporation, Madison USA), is co-transfected to normalize for transfection efficiency. Cell extracts are prepared 48 h after transfection and assayed for firefly and renilla luciferase activity as described by the manufacturer (Dual luciferase reporter assay system, Promega Corporation). All the luciferase values are normalized for renilla luciferase activity.

Example IX: RNA interference experiments

RNA interference (RNAi) is a form of post-transcriptional gene silencing mediated by short double stranded RNAs (dsRNA) that has been described in plants,

nematode, invertebrates organisms and mammalian cell culture ((Ngo, Tschudi et al. 1998) (Vaucheret and Fagard 2001) [Caplen, 2000 #170; Kennerdell, 1998 #171; Timmons, 1998 #172]). However, in plants a transcriptional gene silencing mechanism based on DNA methylation has also been suggested (Wassenegger 2000). DsRNAs have been shown to induce a degradation response in which single stranded RNA complementary to the short dsRNA is rapidly degraded (Montgomery, Xu et al. 1998). RNAi can thus be used to reduce gene expression for instance in whole organisms or invertebrate and vertebrate cell lines (Kennerdell and Carthew 1998), (Elbashir, Harborth et al. 2001), (Caplen, Fleenor et al. 2000). Several methods to introduce dsRNA into cells can be found in the literature. By hand of an example, we describe herein the treatment of *Drosophila* cells with dLgs dsRNA.

Lgs dsRNA preparation

Lgs dsRNA can be made from cDNA or genomic DNA templates, as long as most of the dsRNA corresponds to exon regions. Normally, target regions of 700 to 800 base pair are the most active. However, is known that dsRNAs as short as 200 base pair and as long as 2000 base pairs have potent interfering activities. Both RNA strands can be synthesized simultaneously from a PCR fragment, which contains for instance a T7, SP6 or a T3 promoter on each end. This PCR fragment can be generated by amplification of Lgs cDNA or genomic DNA with 2 primers containing e.g. T7-polymerase binding sites. Primers complementary sequences should be 20 to 24 nucleotides in length with a 22 nucleotides optimum and 60°C optimum Tm. The 5' end of each primer should correspond to e.g. a 27 nucleotides T7 promoter sequence (TAATACGACTCACTATAGGGAGACCAC). The PCR reaction is then performed with a suitable template containing Lgs sequences. Taq polymerase gives the best yields, but another polymerase like Pfu may be used, too. The first 10 cycles should have a 40°C annealing step, followed by 35 cycles with a 55°C annealing step. DMSO can be added to a final concentration of 5% when needed.

Phenol-chloroform extract and ethanol precipitation in NH_4OAc may be used to isolate the PCR template from the reaction mix however other commercially available PCR-purification kit can be used as well. The RNA synthesis reaction can be performed in 50 µl volume with 1 µg of PCR DNA template using an appropriate RNA polymerase. The MEGAscript™ kits from Ambion work very well. The RNA becomes double-stranded during the synthesis reaction. The DNA template can be removed with RNase-free DNAase and the dsRNA can be purified by phenol-chloroform extraction and ethanol precipitation. Typical yields of RNA from 1 µg DNA template are in the 80 to 120 µg range. dsRNA is stored as a NaOAc/ethanol precipitate at -80°C until immediately before use.

The quality of the dsRNA can be monitored by native agarose gel electrophoresis in TBE. Only preparations should be used in which the electrophoretic mobility of most of the RNA is shifted to the mobility expected for dsRNA (very close to duplex DNA mobility) of the appropriate length.

Transfection of Lgs dsRNA into Drosophila S2 cells

S2 cells are propagated in Schneider S2 Drosophila medium (GIBCO) supplemented with 10% FCS. One day before transfection one million cells are seeded into 6 well plates and grown overnight at 25°C. Cells are then transfected using the cationic lipid CellFectine (GIBCO) using an adaptation of the manufacturer's protocol. Briefly, a total of 5 µg DNA and dsRNA is complexed with 20 µl of CellFectine lipid mix in 1.2 ml serum free growth medium (e.g. DES expression medium from Invitrogen, Carlsbad,USA). The complexes are incubate for 15 minutes at RT and then added to the cells from which the normal growth medium has been replaced with 1 ml serum free medium. Four hours later 1.2 ml growth medium supplemented with 30 % FCS is added to the cells. One day after transfection the medium is replaced with fresh medium containing 10% FCS. Cells can be assayed from 2

days after transfection (e.g. for Lgs protein level or for Tcf transcriptional activity).
Similarly, mammalian Lgs expression can be reduced using the method described in (Elbashir, Harborth et al. 2001).

Example X: Search for Lgs homologues

Bcl9, a human protein involved in B-cell lymphoma was identified by searching a public sequence database (http://www.ch.embnet.org/software/aBLAST.html) with fragments of about 500 amino acids of the *Drosophila* Lgs protein. The matrix used was Pam70 and the parameters were set so that repetitive sequences were filtered out. Although the overall homology of Bcl9 and dLgs is very low, they share several short stretches of amino acids with high homology and in the same sequential order (see Figure 7). Local alignments were generated using A WWW server implementation of LALIGN (version 2.0u6319919. The parameters used are: matrix: pam120; gap penalties: -14/-4; alignment 4 edited by hand.
The hlgs-1 gene was found by searching the public high throughput sequence database for predicted coding sequences (cDNA) with homology to the translated sequence of Bcl9 protein fragments (Figure 10). The program used was tblastn, whereas the parameters and matrixes were the same as described above for Lgs. The gene is situated on chromosome 11 and several EST are present in the public human genome databases. Transalation of the predicted cDNA and EST and a first assembly attempt results in a predicted protein containing all the homology domains of figure 7. For instance hLgs-1 has a 54% and 57% amino acids identity with dLgs and hLgs, respectively, in domain 1, and a 23% and 60% amino acid identity, respectively, in domain 2 (data not shown).

Example XI: Rescue of dLgs-/- flies with hLgs/Bcl9 cDNA expression

In order to confirm the functional homology between Drosophila and human Lgs, the human gene was introduced into Drosophila mutant embryos lacking endogeneous dLgs. Specifically, a tub:hLgs/BCL9 transgene was introduced into mutant lgs20F/lgs20F and lgs17E/lgs21L flies (described above). These Lgs mutant flies display larval or pupal lethality. In contrast, flies carrying the tub:hLgs/BCL9 transgene survive to adulthood and are capable of exclosing from the pupa. This demonstrates that despite the low sequence homology hLgs can replace dLgs function in the flies and is thus a true functional homologue.

Example XII: Screening for small molecules inhibiting Lgs-β-Cat or Lgs-Dol1

Several assays are available to test for inhibitors of protein-protein interactions. They can be cell-based or in vitro-based. Cell-based assays are for instance reporter gene assays and yeast or mammalian two hybrid assays. Cell-free assays can be subdivided into heterogeneous and homogeneous assays. In general homogeneous assays are preferred because they avoid washing steps and therefore results in higher throughput compared to heterogeneous assays (e.g. ELISA). Novel homogeneous assay technologies are, for example, the scintillation proximity assay (SPA) (Cook 1996), and fluorescence-based assays such as homogeneous, time resolved fluorescence (HTRF) (Kolb, Burke et al. 1997) and fluorescence polarisation (FP) (Sittampalam, Kahl et al. 1997). By means of an example we describe herein the conditions to screen a chemical library at high throughput for inhibitors of the hLgs-β-Cat interaction using the HTRF technique.

A homogeneous time-resolved fluorescence (HTRF) assay was developed to monitor hLgs/β-Cat binding. This assay employs a histidin-tagged (His-tag) hLgs(300-434) fragment, a GST-fused β-Cat(Armadillo repeat 1-13), and two fluorophore-conjugated detection reagents, XL665-labeled anti-His- and europium cryptate-labeled anti-GST antibodies. The recombinant proteins needed for the assay are produced in BL21 bacteria (e.g. Novagen) and purified over a Nickel column (His-tagged hBcl9) or glutathione beads (GST-β-Cat(Arm-repeats 1-13)). As a negative control a His-tagged hLgs fragment lacking the β-Cat binding domain (hLgs(300-434)Δcoil) was generated. The other reagents and the technical devices needed are commercially available (e.g. by Wallac or Packard instruments). Energy transfer from europium cryptate to the acceptor chromophore XL665 can only occur if the distance between the two molecules is short. Binding of hLgs to β-Cat in binding buffer (20 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM DTT, 1 mM $MgCl_2$, 10 % glycerol, 0.5% NP40, 0.05% BSA) brings the fluorophores into close proximity, allowing fluorescence resonance energy transfer to occur. In the presence of a molecule which inhibits hLgs-β-Cat interaction the distance between donor and acceptor fluorophore is increased resulting in a reduced fluorescence signal. This assay can be up-scaled to work in 384 well plates allowing the screening of several thousand potential inhibitory compounds a day.

Example XIII Use of Lgs homology domain two peptides to inhibit Wnt signaling *in vivo*.

To demonstrate the essential role of the sequence homology domains (HD) of Lgs described in Figure 7 for the propagation of the Wnt signaling pathway, a Tcf-reporter gene assay was performed. In this, HEK293 cells at 50% confluence were plated into 24-well plates and

transfected by a lipofection method. 240 ng of TOPFLASH luciferase reporter plasmid (Upstate biotechnology, New York, USA), 4 ng of pcDNA3-ΔArm, 200 ng of pcDNA3-EGFP-hLgs-peptide and 10 ng of a renilla luciferase reporter plasmid pRL-SV40 (Promega Corporation, Madison USA) were diluted into 25 µl of OPTI-MEM Medium (Life Technologies, Inc.) and combined with 1.2 µl of Lipofectamine (Life Technologies, Inc.) in 25 µl OPTI-MEM. After incubation for 20 min, 0.175 ml of OPTI-MEM was added and the mixtures were overlaid onto monolayers of cells. After culturing at 37°C/5% CO_2 for 6 hr, 0.225 ml of OPTI-MEM containing 20% FCS was added to the cultures. Cell extracts were prepared 48h after transfection and assayed for firefly and renilla luciferase activity as described by the manufacturer (Dual luciferase reporter assay system, Promega Corporation). Small peptides including the HD1 (such as hLgs/Bcl9(199-392) or hLgs/Bcl9(279-392)) strongly inhibit Arm-Tcf transcriptional activity.
Importantly, Lgs HD 2 peptides also inhibit Tcf-driven luciferase activity in SW480 colon carcinoma cells (American Tissue Culture Collection), which have a constitutively active β-Cat due to a mutation in the APC gene (Smith, Johnson et al. 1993) (Figure 15B). As a positive control, a dominant negative hTcf4 (dnTcf4) protein was used (Roose, Huls et al. 1999). Cells were transfected as described above but using Lipofectamine 2000 (GIBCO Life Technologies) instead of Lipofectamine following the manufacturer recommendations.
These results indicate that Lgs peptides can be used for the therapy of diseases characterized by an over-activation of downstream components of the Wnt pathway.

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